REVIEW SUMMARY

SYNTHETIC BIOLOGY

Programming gene and engineered-cell therapies with synthetic biology

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BACKGROUND: Gene and engineered-cell therapies promise new treatment modalities for incurable or difficult-to-treat diseases. First-generation gene and engineered-cell therapies are already used in the clinic, including an ex vivo gene-replacement therapy for adenosine deaminase deficiency, chimeric antigen receptor (CAR) T cell therapies for certain types of leukemias and lymphomas, an adeno-associated virus gene therapy for inherited retinal diseases, and investigational therapies for β-thalassemia, sickle cell disease, hemophilia, and spinal muscular atrophy. Despite these early successes, safety concerns may hamper the broader adoption of some of these approaches: For example, overexpression of a therapeutic gene product with a narrow therapeutic window may be toxic, and excessive activation of T cells can be fatal.

More-sophisticated control over cellular activity would allow us to reliably "program" cells with therapeutic behaviors, leading to safer and more effective gene and engineered-cell therapies as well as new treatments.

ADVANCES: Recent advances in synthetic biology are enabling new gene and engineered-cell therapies. These developments include engineered biological sensors that can detect disease biomarkers such as microRNAs and cell-surface proteins; genetic sensors that respond to exogenous small molecules; and new methods for interacting with various components of the cell—editing DNA, modulating RNA, and interfacing with endogenous signaling networks. These new biological modules have therapeutic potential on their own and can also serve as building blocks for

sophisticated synthetic gene "circuits" that precisely control the strength, timing, and location of therapeutic function. This advanced control over cellular behavior will facilitate the development of treatments that address the underlying molecular causes of disease as well as provide viable therapeutic strategies in situations where the biomolecular targets have been previously considered "undruggable."

Recent publications have demonstrated several strategies for designing complex therapeutic genetic programs by combining basic sensor, regulatory, and effector modules. These strategies include (i) external small-molecule regulation to control therapeutic activity postdelivery, (ii) sensors of cell-specific biomarkers that activate therapeutic activity only in diseased cells and tissues, and/or (iii) feedback control loops that maintain homeostasis of bodily systems. Example therapeutic systems include a genetic circuit that senses two specific cell-surface markers to activate CAR T cells only in the presence of target cancer cells, a circuit that programmatically differentiates pancreatic progenitor cells into insulin-secreting β-like cells, and a gene network that senses the amount of psoriasis-associated cytokines to release immunomodulatory proteins only during flare-ups. These proof-of-concept systems may lead to new treatments that are dramatically safer and more effective than current therapies.

OUTLOOK: Rapid progress in synthetic biology and related fields is bringing therapeutic gene circuits ever closer to the clinic. Ongoing efforts in modeling and simulating mammalian genetic circuits will reduce the number of circuit variants that need to be tested to achieve the desired behavior. The platforms used to test genetic circuits are also evolving to more closely resemble the actual human environment in which the circuits will operate. Human organoid, tissue-on-a-chip, and whole-blood models will enable higher-throughput circuit characterization and optimization in a more physiologically relevant setting. Progress in nucleic acid delivery will improve the safety and efficiency with which therapeutic nucleic acids are introduced to target cells, and new methods for immunomodulation will suppress or mitigate unwanted immune responses. Together, these advances will accelerate the development and adoption of synthetic biology-based gene and engineered-cell therapies. ■
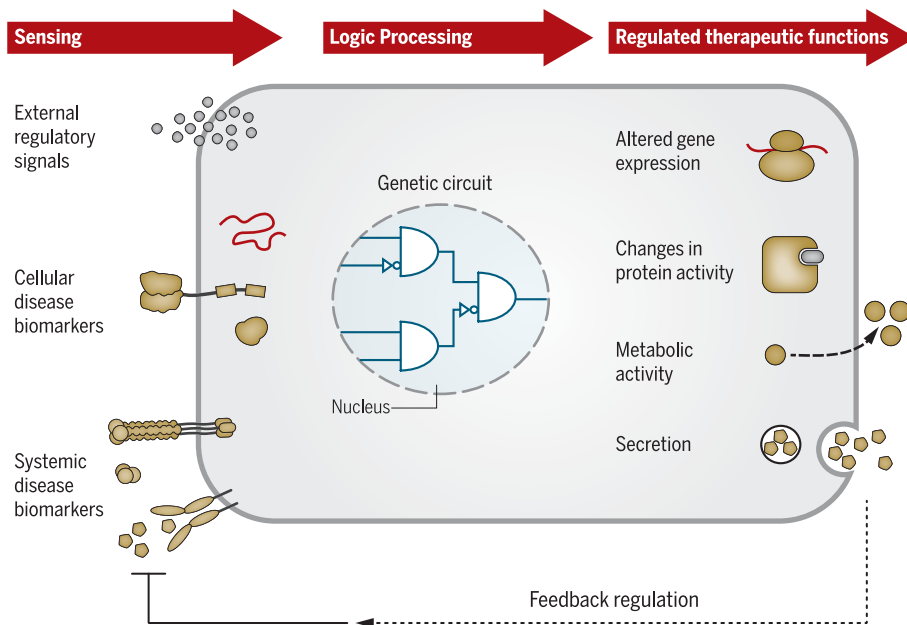
Programming gene and engineered-cell therapies with synthetic biology to improve human health. Genetically encoded therapeutic programs can regulate the dosage, localization, or timing of therapeutic function by sensing and processing externally administered signals as well as cell-specific and systemic disease biomarkers. These synthetic gene networks may lead to gene and engineered-cell therapies that are safer and more effective and that can address a broader class of diseases than current approaches.

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REVIEW

Programming gene and engineered-cell therapies with synthetic biology

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Gene and engineered-cell therapies promise to treat diseases by genetically modifying cells to carry out therapeutic tasks. Although the field has had some success in treating monogenic disorders and hematological malignancies, current approaches are limited to overexpression of one or a few transgenes, constraining the diseases that can be treated with this approach and leading to potential concerns over safety and efficacy. Synthetic gene networks can regulate the dosage, timing, and localization of gene expression and therapeutic activity in response to small molecules and disease biomarkers. Such "programmable" gene and engineered-cell therapies will provide new interventions for incurable or difficult-to-treat diseases.

Gene and engineered-cell therapies use nucleic acids to repair or augment a cell's genetic "program" to change its behavior in a therapeutically useful manner. For example, transducing the hematopoietic stem cells of β-thalassemia patients with a functional β-globin locus may cure their disease (*1*), and genetically modifying a cancer patient's T cells ex vivo with a chimeric antigen receptor (CAR) may allow them to destroy tumor cells when transplanted back in vivo (*2*). These approaches represent a new wave of rational therapeutic design and open exciting new avenues to treat, or even cure, previously intractable diseases (*3*).

However, although gene and engineered-cell therapies are starting to demonstrate promising clinical results, an important limitation of many current approaches is that they provide little control over the strength, timing, or cellular context of the therapeutic effect. This lack of control may hamper broader adoption of these approaches: For example, existing gene therapies usually rely on overexpression of a therapeutic gene product, which may not be appropriate for interventions that have a narrow therapeutic window or require a graded or time-varying response. Clinical trials of engineered cancer-fighting T cells have reported a number of fatal or life-threatening adverse events, including cytokine release syndrome and neurotoxicity related to excessive activation of engineered T cells (*2*). Therapeutics that are activated in response to small molecules or disease biomarkers may prove safer and more effective than current treatments as well as enable new treatments for diseases that are difficult to treat with current approaches.

The field of synthetic biology aims to develop such sophisticated, programmable control over cellular behavior. Synthetic biologists build new biological systems by combining traditional engineering concepts (computer-aided design, modularity, abstraction, feedback control) with new design rules specifically suited to engineering biology (for example, codon optimization and strategies to avoid toxicity caused by the expression of transgenes) (*4*). As the field has developed, synthetic systems have evolved from simple transcriptional regulatory networks in prokaryotes (*5*, *6*) to complex multimodal biological circuits in every branch of life, including mammalian systems both in vitro and in vivo (*7*). Many recent advances have biomedical potential, such as detecting cancer cells by means of their microRNA (miRNA) signature (*8*) and maintaining insulin homeostasis with engineered transplanted cells (*9*). These developments promise a new generation of gene and engineered-cell therapies based on sophisticated synthetic biology methods.

This Review discusses the progress of and prospects for bringing mammalian synthetic biology from the bench to the bedside. We begin by mapping out approaches for controlling cellular behavior at the DNA, RNA, and protein levels, discussing a number of biological modules that might be applied in gene and engineered-cell therapies. Next, we review several strategies that gene circuits can use to control the strength, timing, and context of a therapeutic effect. We conclude by discussing several remaining challenges for the field of mammalian synthetic biology, including better tools for predictive biological design and more clinically relevant testing platforms. Taken together, recent advances promise precise, context-specific control over cellular behavior, leading to new or improved therapies for a host of diseases.

Biological modules: Building blocks for therapeutic circuits

One of the most important principles of engineering, modularity, allows engineers to design complex systems by combining simpler functional units with defined inputs and outputs. These simpler units, or modules, can be designed, tested, and characterized independently before being integrated together. Biological systems can similarly be thought of as a hierarchical connection of many simpler units (*10*), the simplest of which are molecular interactions. For example, transcription can be thought of as a module with two "inputs" (a DNA molecule containing a promoter and a transcription factor) and one "output" (an RNA molecule). These molecular interfaces allow bioengineers to create synthetic modules that interact with endogenous cellular processes, and they support the creation of more-complex synthetic systems by means of the composition of these modules.

Like all abstractions, this definition of biological modularity ignores many important details for the sake of conceptual simplicity. By focusing initially on the modules' inputs and outputs, this abstraction can make designing complex "biological programs" more tractable: The overall desired behavior of a system is expressed as a composite set of logic operations (Fig. 1A), which in turn are decomposed into modules that encode appropriate logical relationships and whose inputs and outputs can be properly connected. For instance, imagine a "second-generation" monogenic gene therapy that allows a clinician to control the strength and timing of transgene expression by administering an appropriate small-molecule drug. The overall behavior of this therapy can be expressed by a simple Boolean AND gate, in which the therapy is only "ON" if both the DNA and the small molecule are present. We begin by decomposing the overall desired system behavior, "DNA AND small-molecule drug → protein," into a set of molecular modules. The required "AND" logic can be implemented in many ways (Fig. 1C): For example, the program's transcription module could be one that requires a small-molecule drug–activated transcription factor for transgene transcription (*11*). Alternatively, we could insert a drug-sensitive ribozyme in the transgene's 5′ untranslated region (UTR), which regulates the transcript's degradation rate (*12*). Choosing an appropriate strategy requires deeper consideration of the clinical requirements, biological dynamics, and cellular context of the eventual therapeutic circuit.

The effective design of gene circuits requires a broad understanding of the available modules, and thus we begin this Review with a survey of synthetic modules that are particularly useful for gene and engineered-cell therapies. The survey groups modules together on the basis of their output, classifying them as directly affecting the abundance or activity of DNA, RNA, or proteins (Fig. 1C). This structure reflects our focus on therapeutic applications, as it is often useful to map a module's functional effect to the molecular basis of a disease. For example, patients with spinal muscular atrophy (SMA) have decreased levels of correctly spliced survival of motor neuron (SMN) mRNA, and therefore, modules that affect mRNA could be utilized. Amounts of the

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functional full-length SMN splice isoform could be restored by increasing pre-mRNA production with a synthetic transcription factor or by targeting a splicing silencer region with a splice-switching oligonucleotide (*13, 14*). For each category of modules, we discuss several recent examples, emphasizing both their utility in engineering synthetic gene circuits and their applicability in a clinical context.

Biological modules that control DNA

Biological modules that act on DNA (Fig. 1C) have the potential to be powerful therapies, because editing a cell's genome can cause permanent changes in its phenotype. For example, targeted DNA cleavage and subsequent repair of gene regulatory elements may one day treat β-hemoglobinopathies (*15, 16*). Although gene-editing tools based on zinc-finger nucleases and transcription activator-like effector (TALE) nucleases have been used in several clinical trials (*17, 18*), recent advances in CRISPR systems are generating excitement because their targets are specified with a guide RNA (gRNA) by using simple base-pairing rules instead of laborious protein engineering. CRISPR-enabled gene editing is already generating encouraging results in animal models (*19–21*); for example, Tabebordbar *et al.* used a Cas nuclease from *Staphylococcus aureus*, SaCas9, to excise a mutated intron in the *mdx* mouse model of Duchenne muscular dystrophy. The CRISPR system has also been extended to enable direct base editing (*22, 23*) and targeted DNA demethylation (*24, 25*).

Gene-editing tools, including Cas nucleases, can also be targeted to exogenous DNA that encodes the synthetic gene circuit itself as part of the biological program or as a safety mechanism. For example, a recent effort used a Cas9 system as a DNA-based memory recording device (*26*). The Cas9 transgene was placed under control of a nuclear factor κB (NF-κB)–responsive promoter, and the nuclease was targeted to the DNA sequence encoding the gRNA. When cells containing this memory device were implanted in vivo, the number of mutations in the gRNA sequence reflected the intensity and duration of NF-κB–mediated inflammation. A similar self-targeting strategy might be used as a "self-destruct" component of a gene circuit, enabling the destruction of DNA-encoded therapies if unintended adverse effects arise or when the therapy is no longer required. For example, our group created a Cas9-based "safety switch" (*27*), where Cas9 cleaves circuit-related DNA upon the addition of a small molecule. The circuit is there-fore only expressed in cells where the circuit DNA is present "AND NOT" the small molecule–induced gRNA-loaded Cas9 (Fig. 1C).

Biological modules that control RNA

Many different cellular processes regulate the production, stability, conformation, splicing, and translation of RNA (*28*). The broad range of processes that involve RNA make it a compelling target for engineering efforts, and dysfunctions in these processes make synthetic systems that interact with RNA of considerable therapeutic interest. For example, a synthetic splice-switching oligonucleotide (Fig. 1C) was approved by the U.S. Food and Drug Administration (FDA) for the treatment of SMA (*14*), and a synthetic small interfering RNA (siRNA), which targets transthyretin (TTR) mRNAs for degradation, demonstrated strong clinical benefits in a trial for patients with TTR amyloidosis (*29*). These successes suggest that more-sophisticated synthetic biological modules that control the abundance and activity of RNA may have broad therapeutic potential.

One way to control the abundance of RNA is by regulating its transcription from DNA. Synthetic transcription modules (Fig. 1C) in mammalian systems have typically been built by fusing DNA binding domains with activation and repression domains (*30*). For example, Zhang *et al.* created synthetic transcriptional activators by attaching the potent synthetic activation domain, VP64, to TALE DNA binding domains designed to target sequences upstream of endogenous genes (*31*). Customizing the DNA binding specificity of synthetic transcription factors has become even easier with the development of catalytically "dead" dCas9, which still binds DNA but does not cleave it. For instance, dCas9-based transcriptional activation systems designed to target the 5′ long terminal repeat of dormant HIV proviral DNA have been used to induce HIV in cell culture models of latency (*32*).

Additionally, the abundance, availability, and translational or catalytic activity of RNA can be modified directly by interactions with other biomolecules. For example, Chen *et al.* created a
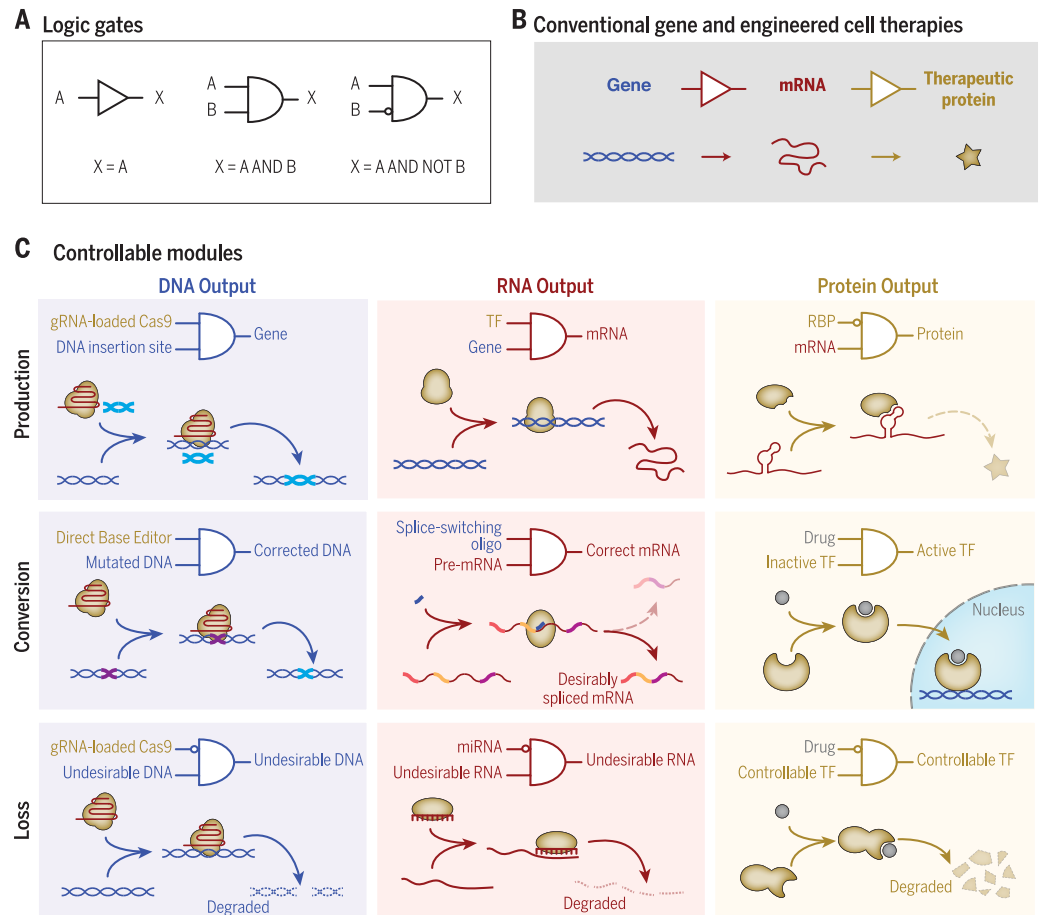
Fig. 1. Building blocks for therapeutic programs. (**A**) Logic gates with inputs (A and/or B) and outputs (X) can be used to represent molecular processes and reactions. (**B**) Conventional gene and cell therapies require just one exogenous molecular input and lack precise control over the output. Such modules function as buffer gates (i.e., control devices whose output levels correspond to their input levels), because the RNA output will be produced in any cell that the DNA input is delivered to and the therapeutic protein will be translated correspondingly. (**C**) Engineerable modules can regulate the production, conversion, or loss of specific DNA (blue), RNA (red), or protein (yellow) species by using more than one molecular input. TF, transcription factor; RBP, RNA-binding protein.

synthetic RNA device that deactivates an adjacent ribozyme upon binding theophylline (*12*). When inserted into the 3′UTR of a transcript encoding interleukin-15 (IL-15) in a mouse cytotoxic T cell line (CTLL-2), the theophylline switch implements a logic AND module: Only in the presence of both the RNA transcript and theophylline is IL-15 expressed. This enabled control over IL-15–mediated proliferation of these T cells in mice to improve clonal expansion after adoptive transfer. More recently, orthologs of the RNA-guided RNA-targeting CRISPR-Cas effector Cas13 have been used in mammalian cells to both knock down and directly edit endogenous mRNA transcripts with high specificity (*33, 34*). Such programmable RNA-binding proteins enable precise posttranscriptional regulation of endogenous RNAs, which has exciting therapeutic implications.

Finally, just as siRNAs can be used to target endogenous mRNAs for degradation (Fig. 1C), synthetic biological modules can regulate RNA abundance by interfacing with endogenous host RNA-degradation machinery. For example, engineered mRNAs containing miRNA target sites in their 3′UTRs can be used as miRNA sensors because their abundance (and the abundance of the translated protein) is inversely related to the amount of cognate miRNA in the cell. This approach was used to control the replication of an engineered herpes simplex virus 1 (HSV-1) oncolytic virus by inserting miR-124 target sites into the 3′UTR of an essential viral early gene (*35*). Because miR-124 is expressed in neurons, but not glioblastoma cells, viral replication occurred in cancerous cells but not in healthy neuronal tissue.

Biological modules that control proteins

Proteins are a biochemically diverse molecular species that transduce information, catalyze synthesis and conversion of other biomolecules, and serve as structural components inside and outside the cell. Because of this functional diversity, clinicians have been able to use protein "biologics" (i.e., complex drugs manufactured in and isolated from living cells) to treat diseases, including autoimmune, metabolic, and cardiovascular disorders as well as cancer (*36*). Synthetic biology promises to improve upon these therapies by providing precise control over the abundance, localization, and activity of therapeutic proteins, making them safer and more effective.

Because most gene circuits express proteins from RNA, any of the strategies discussed above for controlling RNA abundance can also be used to modulate protein levels. Additionally, an mRNA's translation can be influenced by its interactions with other proteins and small molecules. For example, the archaeal ribosomal protein L7Ae binds the box C/D kink turn (K turn) and related K-loop RNA motifs, and this binding has been shown to strongly inhibit translation of the RNA (*37, 38*). This module therefore produces a protein output when the logic function "mRNA AND NOT L7Ae" is true (Fig. 1C). Endogenous RNAs can be modulated in this way as well, by means of program-

mable RBPs such as the Pumilio/fem-3 mRNA-binding factors (PUF) (*39*), pentratricopeptide repeat (PPR) proteins (*40*) and, more recently, RNA-targeting Cas effector proteins (*33, 41–46*). Together, these new tools are rapidly expanding the list of RNAs whose translation can be controlled by engineered systems.

Finally, a protein's abundance and activity can be modulated by fusing it to domains that respond to small molecules (Fig. 1C). For example, a number of modular degradation domains are stabilized by small-molecule ligands, allowing for direct external control of protein levels (*47–49*). Banaszynski *et al.* fused one of these domains to the cytokine tumor necrosis factor–α (TNF-α) and then used the small-molecule ligand Shield-1 to control the strength and timing of the cytokine's expression in mice. The fusion protein was expressed from a strain of vaccinia virus that preferentially replicates in tumor cells. When Shield-1 was administered 3 days after viral delivery, TNF-α expression was localized primarily to the tumor cells, demonstrating control over both the localization and timing of an otherwise toxic gene product (*50*). Another recent example is the synthetic thyroid hormone homeostasis system developed by Saxena *et al.* (*51*), which uses the hormone-binding domain of human thyroid receptor alpha fused to a DNA binding domain to activate a reporter transgene in response to thyroid hormone. When they replaced the reporter transgene with a thyroid-stimulating hormone receptor antagonist, the construct was able to restore thyroid hormone homeostasis in a mouse model of Graves' disease. This last example of a prototype therapeutic gene circuit uses feedback to modulate the level of its therapeutic output: The output of the circuit decreases thyroid activity, which reduces thyroid hormone amounts that are, in turn, the input to the circuit. This negative feedback is one strategy that therapeutic gene circuits can use to control the strength, timing, and context of their therapeutic output. Such control strategies, and gene circuits that implement them, are the subject of the next section of this Review.

Genetically encoded therapeutic programs

Although recent years have witnessed an increase in the number of successful gene and engineered-cell therapy trials for various diseases, more-precise regulation of the dosage, localization, or timing of a treatment's therapeutic activity may lead to improved safety and efficacy profiles for existing treatments as well as enable new modes of therapeutic intervention. For instance, implanted cells engineered with "prosthetic" gene circuits may one day treat autoimmune disorders by sensing systemic disease-associated biomarkers and secreting immune-modulating proteins in response (*52*). In this section, we describe three synthetic biology strategies to more precisely control gene and engineered-cell therapies. In the first strategy, a synthetic gene circuit's activity is externally modulated by small molecules, affording a clinician precise control over the intensity and timing of therapeutic functions. In the second

strategy, gene circuits sense intracellular and extracellular biomarkers to spatially restrict therapeutic activities to diseased cells and tissues. Finally, in the third strategy, gene circuits use feedback control loops to adaptively modulate the activity levels of therapies to treat diseases caused by disrupted homeostasis. For each strategy, we examine several recently reported gene circuits, exploring how therapeutic requirements drive the design of synthetic biology solutions. Together, these examples highlight how synthetic biology could pave the way to safer and more-effective gene and engineered-cell therapies.

Small molecule–based regulation of gene and engineered-cell therapies

Successful clinical translation of gene and engineered-cell therapies would be facilitated by the ability of a clinician to control the activity of a therapy once it has been administered to a patient. In some settings, external control can decrease the likelihood that excessive activity of an engineered-cell or gene therapy results in harm to the patient. For example, serious adverse events have been recorded in a number of recent CAR T cell cancer immunotherapy trials (*2*). CAR T cell therapy is a potent new cancer treatment in which a patient's T cells are harvested, genetically engineered with a CAR against a tumor antigen, expanded, and reinfused into the patient. Unfortunately, in some patients, through a not yet fully characterized process linked to excessive activation of the infused cells, CAR T cell therapy can cause severe neurotoxicity or cytokine release syndromes that have proved fatal (*2*).

One possible way to address such problems is to engineer gene circuits whose activities are regulated by the administration of small molecules. Gene and engineered-cell therapies may persist in the body for a relatively long period of time, whereas small molecules typically have short half-lives in vivo and thus can be used to precisely control the activities of gene and engineered-cell therapies. For example, to enable external control over cytotoxic T cell function, Wu *et al.* created a CAR whose activation was dependent on a rapamycin analog (rapalog) (*53*). CAR proteins are typically composed of a single-chain variable fragment (scFv) fused to activating domains of the T cell–receptor CD3ζ intracellular domain and costimulatory domains such as CD28 or 4-1BB (Fig. 2A). Wu *et al.* modified the original CAR to develop an ON-switch CAR system consisting of two modular transmembrane proteins: one containing the extracellular scFv domain, a 4-1BB costimulation domain, and an FKBP domain; and the other composed of the CD3ζ T cell activator, a second 4-1BB domain, and an FRB domain. The FKBP and FRB domains interact to create a complete receptor only when rapalog is present (Fig. 2A). Small molecule–dependent activation of the ON-switch CAR T cell was demonstrated in vitro using a cell-killing assay, as well as by tumor clearance in a xenograft mouse model of CD19-positive lymphoma, establishing the proof of concept of a titratable CAR T cell system.

The key to this circuit's function is the careful design of the split CAR. In particular, the CAR's activity is controlled by rapalog posttranslationally. This allows for a timely response to the small molecule and might allow the CAR T cells to be shut down rapidly in patients experiencing cytokine release syndrome or neurotoxicity. This strategy is advantageous over a design in which a small molecule controls the transcription of a CAR: In such a scenario, full activation of the CAR T cell would be delayed by the transcription and translation processes necessary to produce the receptor, and shut off of T cell activity would be slow because of the time required for the CAR protein to be degraded.

Although small-molecule control over gene circuit activity has obvious safety benefits, the same strategy can also be used more broadly to control therapeutic gene circuit behavior to facilitate new modes of therapeutic interventions. For example, Saxena et al. developed a synthetic gene network that uses increasing concentrations of vanillic acid (VA) to differentiate pancreatic progenitor cells into insulin-producing β-like cells (54) (Fig. 2B). Saxena et al. use discrete concentrations (zero, moderate, and high) of VA to sequentially establish three distinct patterns of gene expression, resulting in an OFF-ON-OFF pattern for Ngn3; an ON-OFF-ON pattern for Pdx1; and an OFF-OFF-ON pattern for MafA, which encode three transcription factors that drive cell differentiation. Two different VA sensors enable the circuit's concentration-dependent response: MOR9-1, which is activated by VA, and VanA₁, which is inhibited by high concentrations of VA. Plasmids encoding the circuit were transfected into pancreatic progenitor cells, which were differentiated from induced pluripotent stem cells (iPSCs) by using growth factors and small-molecule inducers. In the absence of VA, circuit-containing pancreatic progenitor cells express endogenous Pdx1, but no genes from the circuit (Pdx1: ON, Ngn3: OFF, MafA: OFF). At moderate concentrations of VA, the odorant receptor MOR9-1 is activated, and this in turn generates moderate concentrations of activated endogenous CREB1. Activated CREB1 binds the highly sensitive P$_{CRE}$ promoter, inducing expression of the synthetic transcription factor VanA₁, which in turn activates transcription of both Ngn3 and a synthetic miRNA against endogenous Pdx1 mRNA (Pdx1: OFF, Ngn3: ON, MafA: OFF). This drives differentiation of the pancreatic progenitor cells into endocrine progenitor cells. Finally, at high concentrations of VA and, therefore, high levels of activated CREB1, the less sensitive P$_{CREm}$ promoter is activated and induces Pdx1 and Maf1, while VanA₁ is inhibited by the high concentration of VA. Inhibition of VanA₁ stops induction of Ngn3 and Pdx1 miRNA (Pdx1: ON, Ngn3: OFF, MafA: ON), producing β-like cells. By changing the concentration of one exogenous molecule, three different gene expression signatures are generated, guiding the stepwise differentiation of pancreatic progenitor cells into β-like cells.

An important feature of this lineage-control network is the tight integration between the synthetic gene network and endogenous machinery for transmitting information and effecting changes to cell state. The VA-activated G protein–coupled receptor MOR9-1 is expressed from a transgene, but it transmits information to the rest of the synthetic gene circuit by means of endogenous species: a G protein, adenylyl cyclase, kinase, and transcription factor. This takes advantage of the signal-amplification properties of the endogenous signaling network (55). Additionally, the three circuit-encoded effector genes that drive cell differentiation, Ngn3, Pdx1, and MafA, also drive transcription from their endogenous loci. These feedback loops, initially activated by the synthetic gene network, serve as signal amplifiers and help the combined gene network achieve the intermediate states that are necessary to drive the pancreatic progenitor cell's differentiation.

Even though the synthetic gene circuit that Saxena et al. describe operates in vitro, circuits that guide multistage (trans)differentiation by using discrete concentrations of a single small molecule may be useful for in vivo therapeutic applications as well. For instance, there is continued interest in using cell-based therapies to repair damaged or diseased tissues in situ, but engraftment of immature cells into existing structures remains a challenge (56). Such therapies generally involve creation of patient-specific PSCs, then implanting them into damaged tissue. Engineering these cells with a gene circuit that directs an engineered–stem cell's differentiation in vivo could
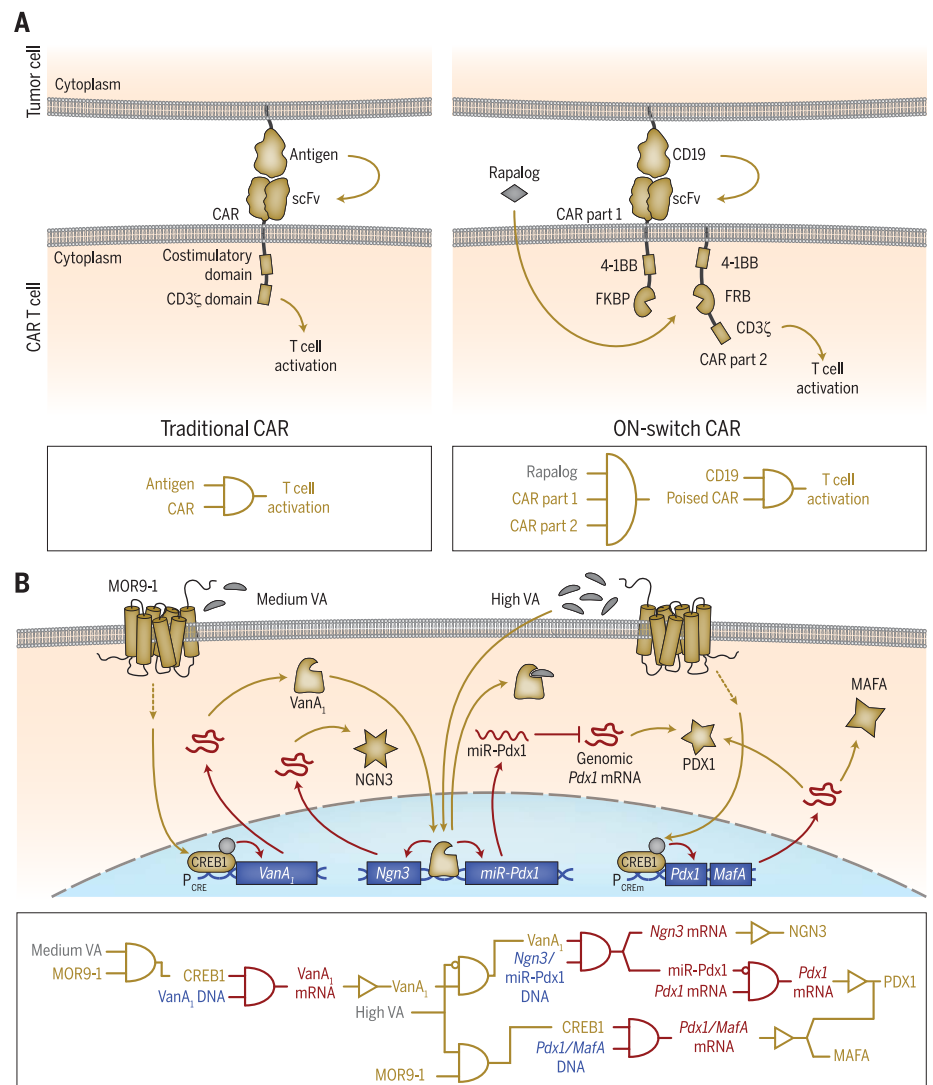
Fig. 2. Small-molecule regulation enables control over strength of therapeutic activity and facilitates new applications. (**A**) Traditional CARs are activated when the T cell encounters a target antigen. ON-switch CARs respond to antigens only when a small molecule, such as rapalog, is administered. (**B**) The pancreatic progenitor-to–β-like cell differentiation circuit is controlled by VA. Increasing levels of VA establish three different gene-expression profiles for the transcription factors PDX1, NGN3, and MAFA to drive differentiation. The final concentration of PDX1 is a summation of translation from two mRNA sources akin to a wired-OR operation in electronic logic circuits. Dashed arrows indicate multiple steps. The same drawing conventions are used as in Fig. 1.
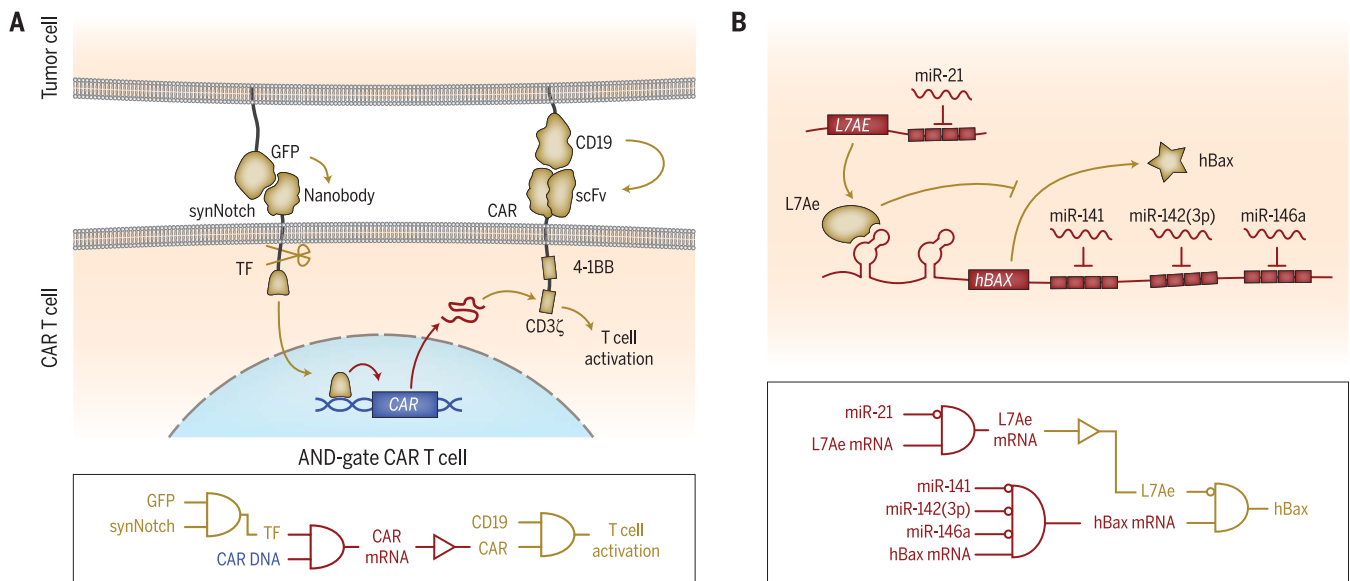
Fig. 3. Genetically encoded therapeutic programs incorporate cell-specific biomarkers for localized activity. (**A**) In an AND-gate CAR T cell, the activation of a synNotch receptor by a first antigen induces the expression of a CAR, which in turn is activated by a second antigen to ultimately activate the T cell. (**B**) RNA-encoded miRNA-classifier circuit selectively kills cancer cells characterized by high levels of miR-21 and low levels of miR-141, miR-142(3p), and miR-146a. The same drawing conventions are used as in Fig. 1.

improve the likelihood that it engrafts successfully. This strategy would be particularly powerful when combined with ex vivo gene editing for monogenic disorders: For example, Duchenne muscular dystrophy could potentially be treated by (i) generating iPSCs from a patient, (ii) correcting the mutated *DMD* gene, which encodes dystrophin, and then (iii) implanting the iPSCs into the patient's muscles and directing their differentiation into mature myofibers. For transdifferentiation, an analogous approach might involve delivering a similar multistage gene circuit to damaged tissues in vivo, which could guide the direct and efficient conversion of one cell type to another. Activating such a circuit in specific cell types is the subject of the next section.

Sensing biomarkers for localized therapeutic activity

Circuits that use endogenous cellular biomarkers to regulate their activity can enable gene and engineered-cell therapies to be more context specific, activating only in the proper cellular environment. This additional specificity can increase potency and reduce off-target activation, making gene and engineered-cell therapies safer and more effective.

CAR T cell–based cancer immunotherapy, discussed above, is a particularly ripe target for biomarker-based gene and engineered-cell therapies. Because an antigen targeted by a CAR is rarely expressed exclusively on tumor cells, T cell activation is sometimes observed in tissues other than the tumor, leading to adverse events in clinical trials. For example, leukemia patients who were administered autologous T cells engineered with CARs against CD19 experienced cancer remission but also long-term depletion of normal CD19$^+$ B cells (*57*). The engineered effector cells in these trials are potently activated by the tumor antigen, but, if their activation could be made more specific, they may serve as the basis for safer therapeutics.

One approach to improving the specificity of engineered CAR T cells is to make them recognize multiple antigens instead of just one. Roybal *et al.* did so by engineering a CAR T cell system that could recognize two independent antigens (*58*), which was based on a synthetic Notch juxtacrine sensor developed by Morsut *et al.* (*59*). The native Notch receptor recognizes a cognate ligand and releases an intracellular domain that activates endogenous gene expression. Morsut *et al.* replaced both the extracellular ligand-binding domain and the intracellular transactivation domain, creating a synthetic Notch receptor (a "synNotch") whose signaling is orthogonal to native cellular machinery. Roybal *et al.* then used the synNotch receptor platform to engineer CAR T cells with improved specificity (*58*). Their system is composed of several modules: (i) a synNotch receptor, which binds to a tumor antigen and releases a synthetic transactivator; (ii) the DNA encoding a CAR, which is activated by the synNotch transactivator; and (iii) the CAR itself, which binds to a second tumor antigen and activates the T cell. This results in a very specific AND gate: Only T cells presented with both the synNotch ligand and the CAR ligand are activated (Fig. 3A). As a proof of concept, Roybal *et al.* built a synNotch sensor for green fluorescent protein (GFP) and a CAR specific to CD19 (*58*). When they implanted a CD19- and GFP-expressing xenograft into mice and injected their engineered T cells, the tumor was cleared. Notably, the AND-gate T cells did not elicit a response against tumor cells that only express GFP or CD19, demonstrating the specificity of the engineered T cells.

The dual-antigen CAR T cell system is a promising platform for personalized medicine because both the synNotch receptor and the CAR can be engineered with virtually any ligand-binding domain. In the future, it may be possible to engineer T cells that precisely target a patient's tumor by using synNotch receptors and CARs or T cell receptors that recognize specific cell-surface markers or major histocompatibility complex epitopes expressed on that patient's tumor cells (*60*). There are also opportunities to add other effectors to the output of the circuit: Because synNotch can activate any exogenous gene, it would be straightforward to expand the circuit with other modules that, for example, modulate the properties of T cells. Indeed, in a recent publication, Roybal *et al.* engineered synNotch T cells that express the T helper type 1 (T$_H$1) specific transcription factor T-bet upon recognition of the CD19 antigen (*61*). These synNotch T cells differentiated into interferon-γ (IFN-γ)–secreting T$_H$1 cells when cocultured with leukemia cells ectopically expressing CD19 but not when cocultured with control leukemia cells.

There are biomarkers besides cell-surface antigens that can be used to distinguish cancer cells from healthy tissues. Intracellular biomarkers, such as miRNAs, can also be detected by gene circuits, providing specificity to broadly cytotoxic anticancer mechanisms that may otherwise have considerable off-tumor activity. In this vein, in collaborations with the laboratories of Benenson, Saito, and Xie, we have created several "cancer-classifier" circuits that use endogenous miRNA expression signatures to distinguish between HeLa cells and healthy cells and activate apoptosis selectively in malignant HeLa cells, while sparing surrounding cells. The original circuit was built using plasmid DNA by Benenson and our group

(8) and was subsequently modified by Lapique and Benenson to reduce leaky output expression by introducing a recombinase-mediated delay in expression of the toxic load (62). In a related project, with Xie's group, we demonstrated HeLa and HEK (human embryonic kidney) cell classification using a cross-repressed TALE repressor circuit (63). This circuit architecture decreased leaky expression and improved the signal-to-noise ratio of cell-type classification. More recently, we implemented another version of the classifier based on the original circuit design (8) but which used only posttranscriptional regulation (Fig. 3B) (64). This allowed us to encode the circuit entirely in synthetic mRNA, a safer therapeutic modality compared to DNA (65). The RNA-encoded circuit consists of mRNA for the RNA-binding protein L7Ae with target sites for miR-21 in its 3′ UTR region and a second mRNA encoding the proapoptotic hBax protein regulated by an upstream K-turn motif and followed by target sites for miR-141, miR-142(3p), and miR-146a. L7Ae binds the K-turn motif and represses expression of hBax. Thus, hBax is expressed only in cells with high levels of miR-21 and low levels of miR-141, miR-142(3p), and miR-146a, a signature specific for HeLa cells. When this mRNA-encoded circuit was transfected into a coculture of HeLa and HEK cells in vitro, the circuit induced apoptosis specifically in HeLa cells.

Several features make miRNA sensing a particularly attractive strategy for designing cell-type classifier circuits. First, current sequencing methods make holistic comparisons of miRNA signatures from different tissues rapid, inexpensive, and reliable. Second, miRNA-sensing modules are easy to design, function potently, and can be combined in tandem to create more-complex logic. One caveat is that miRNA abundance does not always correlate well with miRNA-sensor activity (66). Thus, experimental verification of sensor libraries may be critical for predictable and accurate design of cell type–classifier circuits.

Feedback control for augmented homeostasis

Gene circuits that sense and respond to disease biomarkers by means of feedback loops can regulate therapeutic functions so that they are activated only at the right intensity and time. Such a feature could be particularly beneficial when systemic modes of interventions are used for treatment of disorders related to disrupted homeostasis (67). For example, diet-induced obesity may be treated with this approach. Although the primary treatment is a change in the patient's lifestyle and dietary habits, pharmacological and surgical interventions can assist weight loss by suppressing appetite and reducing fat absorption. One such intervention is treatment with pramlintide, an analog of amylin, which aids in blood glucose regulation and promotes satiety. Pramlintide is approved by the FDA for the treatment of type 1 and type 2 diabetes in patients who use mealtime insulin, but it has also been investigated as an adjunct to lifestyle intervention in obesity treatment (68). Because pramlintide is a peptide,

it can be synthesized via a transgene, which makes it an attractive effector module for a gene circuit to treat diet-induced obesity.

To enable autonomous dosing of pramlintide, Rössger et al. created a feedback loop that coupled its expression to a sensor for an appropriate metabolite. They built such a sensor, dubbed the lipid-sensing receptor (LSR), by fusing the ligand-binding domain of peroxisome proliferator-activated receptor alpha (PPARα) to the phloretin-responsive repressor TtgR (Fig. 4A) (69), which allowed the LSR to bind to a synthetic promoter containing the TtgR operator. The PPARα subunit recruits transcriptional coactivators in the presence of fatty acids, and corepressors in their absence, expressing the transgene strongly in the ON state but abolishing its expression in the OFF state. The DNA binding domain TtgR provides another level of control because its binding to the TtgR operator sequence is repressed by phloretin, an apple-derived small molecule found in many cosmetics. The small-molecule control could serve as an external way to tune system response or abrogate circuit activity. Thus, the LSR forms a logical two-input AND gate with one inverted input, in which the transgene under LSR control is active only when lipids are present but phloretin is absent. In cell culture, this sensor was highly sensitive to exogenous fatty acids, with transgene expression increasing over 100-fold in response to some treatments, such as with rapeseed oil.

Delivery is one of the major obstacles in translating such advances into clinically useful therapeutics. To deliver their prosthetic gene circuit, Rössger et al. (69) engineered the human fibrosarcoma cell line HT-1080 to express LSR and LSR-controlled pramlintide transgene, then encapsulated the cells in alginate–poly-L-lysine beads and injected them intraperitoneally into mice. The alginate encapsulation protects the implanted cells from the host immune response but provides them with access to host metabolism. When obese mice fed a high-fat diet were implanted with the cells, they showed high concentrations of circulating pramlintide and corresponding decreases in blood fat, food intake, and body weight. Prosthetic gene circuits have also been developed to regulate urate (70), blood pressure (71), blood pH (72), thyroid hormones (51), enterohepatic signaling (73), blood glucose (9), and insulin (74).

In addition to metabolic disorders, feedback control of therapeutic gene circuits is also appropriate for chronic diseases that flare up occasionally but for which prophylactic treatment has safety concerns. One example is psoriasis, a common autoimmune disorder that causes inflamed skin lesions and whose comorbidities include psoriatic arthritis, Crohn's disease, metabolic syndrome, and cardiovascular disease (75). The inflammation characteristic of psoriasis is caused by overexpression of cytokines such as TNF-α and IL-22. Existing therapies include antibodies against TNF-α or T_H1- and T_H17-related cytokines as well as various oral or topical treatments, but long-term suppression of the immune system is associated with side effects such as infection (76). Recent phase 2 trials of anti-psoriatic and anti-

inflammatory cytokines IL-4 and IL-10 have shown efficacy in treating psoriasis (77, 78), but the short half-lives of these compounds means that they require almost continuous administration to be efficacious.

To address these challenges, Schukur et al. used a feedback-regulation strategy to engineer a gene circuit that senses TNF-α and IL-22 and drives the expression of IL-4 and IL-10 (Fig. 4B) (52). The sensing half of the circuit shares a similar "serial sensor" design with the synNotch CAR: The endogenous TNF-α receptor activates expression of an IL-22 receptor IL-22RA by means of an endogenous NF-κB signaling cascade; the IL-22RA transgene then senses IL-22 and communicates the signal to the nucleus through an endogenous JAK-STAT (Janus kinase–signal transducers and activators of transcription) cascade. Finally, synthetic STAT3-responsive promoters activate expression of IL-4 and IL-10. The circuit therefore encodes a logical two-input AND gate: The therapeutic outputs (IL-4 and IL-10) are expressed only in the presence of TNF-α and IL-22. In a cultured human-cell assay, the activity was reversible, with production of IL-4 and IL-10 falling after the withdrawal of TNF-α and IL-22, which is a precondition for reacting to changing amounts of pathological cytokines during a psoriatic flare-up. HEK-293T cells engineered with the homeostatic circuit were encapsulated in alginate–poly-L-lysine and injected intraperitoneally into mice, where the prosthetic gene circuit successfully controlled inflammation caused by topical application of imiquimod, a common model for psoriatic lesions. The sensors also responded to cytokines in the blood of psoriasis patients, suggesting that they are sensitive enough to detect circulating TNF-α and IL-22 in humans.

Perspective

Synthetic biology is poised to improve gene- and engineered-cell–based treatments for many diseases by providing precise control over the intensity, timing, and context of therapeutic intervention. Synthetic biology–inspired modules such as safety-switches and gene editing technologies are being introduced to clinical trials, and more-sophisticated gene circuits may one day enable advanced therapies like direct in vivo transdifferentiation. However, although complex synthetic systems have been demonstrated by a growing number of proofs of concept in the lab, challenges remain in developing synthetic biology–enabled therapies. This section explores several of these challenges, including designing synthetic gene networks that meet specified performance goals, translating them from in vitro testing environments to an in vivo therapeutic context, and delivering them efficiently into the patient. We also discuss recent advances in all three areas, which together are moving synthetic biology closer to the clinic.

Despite synthetic biology's explosive development in the past decade, it is still challenging to build gene circuits that behave as anticipated (79), often requiring many design-build-test iterations before a synthetic gene network meets its performance goals. One common reason is
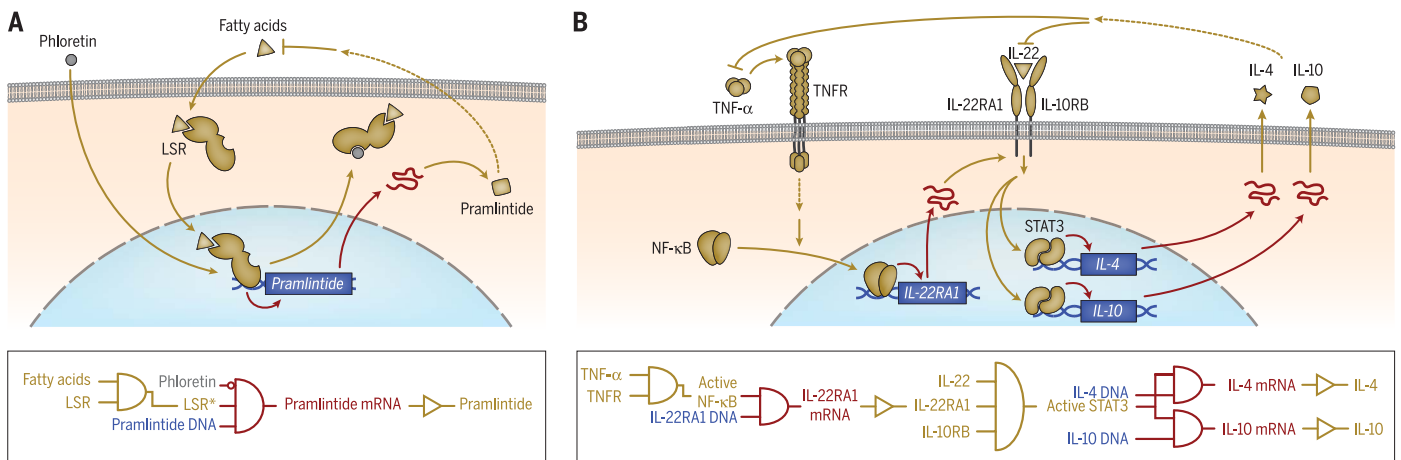
Fig. 4. Gene circuits that use feedback regulation to sense systemic biomarkers and secrete systemically acting effector molecules enable homeostasis. (**A**) A closed-loop circuit to treat obesity responds to fatty acids and produces pramlintide to slow gastric emptying, reduce glucagon, and modulate satiety. (**B**) A cytokine converter circuit to treat psoriasis responds to inflammatory signals TNF-α and IL-22 and produces anti-psoriatic and anti-inflammatory cytokines, IL-4 and IL-10, respectively. Dashed arrows indicate multiple steps. The same drawing conventions are used as in Fig. 1.

unaccounted for context effects: Genetic circuits operate using host-cell resources, which vary from cell to cell and form a finite pool from which all cellular processes (both native and engineered) must draw. Resource competition and other contextual effects can lead to modules that have different behavior depending on other modules in the circuit and circuits that have different behavior depending on the cell type in which they operate (*80*). Characterizing genetic modules in multiple cell types or specific contexts of interest as well as in combination with different modules might help to better account for this context sensitivity when designing larger circuits and systems. In parallel, by developing a deeper understanding of how cellular environments influence the behavior of synthetic modules, bioengineers may design modules and circuits that are better insulated from the cellular context.

One manifestation of context sensitivity is the poor agreement observed anecdotally between circuit performance in vitro and in vivo. Mammalian gene circuits are typically developed in cell lines cultured in artificial environments, which facilitates rapid testing of many circuit variants. Although these culture systems are experimentally tractable, they do a poor job recapitulating the heterogeneous, dynamic in vivo environment in which a therapeutic circuit must ultimately operate. For instance, differences in intracellular biomolecule amounts between cell lines and primary cells (*81*) could be debilitating for synthetic gene circuits whose proper function depends sensitively on the concentrations of their modules' inputs.

Bridging this gap in circuit behavior may be possible using in vitro test systems that more closely resemble the environment inside the body. Promising technologies include organoids, which are three-dimensional "organ buds" grown in vitro, and "organ-on-a-chip" systems where cells are grown in microfluidic systems that mimic tissue

properties. Both of these platforms have shown utility in emulating disease pathologies (*82*, *83*). For example, Ogawa *et al.* created cholangiocyte organoids from iPSCs of cystic fibrosis (CF) patients that recapitulated important aspects of the CF disease phenotype (*84*). Whereas organoids derived from normal iPSCs could regulate cyst swelling through cystic fibrosis transmembrane conductance regulator (CFTR)–mediated fluid transfer, this capability was lost in organoids derived from CF patients' cholangiocytes. The researchers then demonstrated that cyst swelling could be rescued in the diseased organoids by modulators of CFTR. Such organoids may one day facilitate testing of therapeutic synthetic gene circuits in a setting that closely mimics relevant disease pathologies. Another approach might be to characterize genetic circuits embedded in engineered "designer cells" by using human whole-blood culture systems (*52*, *85*). These systems simulate the environment that the engineered cells will be exposed to in a patient, helping to more accurately predict their performance in vivo. For example, engineered HEK-293T cells encapsulated in alginate and cocultured with whole blood were able to respond to the TNF-α produced by primary immune cells stimulated with bacterial lipopolysaccharides (*85*). The continuing development of such platforms will enable high-throughput circuit characterization and optimization in more physiologically relevant settings.

A complementary approach to address gene circuits' unwanted context sensitivity is to develop biological modules and circuit designs that are better insulated from cellular context, for example, by minimizing spurious interactions (crosstalk) with other molecular species or by reducing reliance on host factors and processes. Such modules and designs should be easier to model computationally and may better maintain their behavior as they are moved from model systems to in vivo use. One design technique to achieve this is to

import modules and molecules from other organisms that are expected to have minimal crosstalk with native mammalian molecular networks. For example, a modified form of the *Escherichia coli* transcription factor TetR and its cognate DNA sequence have found broad use in mammalian systems (*11*). Although this module relies on endogenous transcription machinery, there is minimal nonspecific interaction between TetR and other DNA sequences in mammalian cells, which allows for some degree of logical insulation of the module from the cellular context. Another design strategy is to use modules whose interaction partners can be controlled more rationally, such as CRISPR-Cas and RNA-interference modules whose binding is based on Watson-Crick base pairing; although in both cases, off-target binding effects are still being studied.

Both of these approaches to building more–predictable gene circuits can be supported by advances in computer-aided design tools. Better software for designing and simulating biological circuits could reduce the number of circuit variants that need to be built and tested, leading to faster and cheaper development of synthetic-biology therapies. Early efforts to simulate the behavior of synthetic gene networks relied on mechanistic models that captured each species' production, transport, binding, etc. (*86*), but these models' predictive power decreased as the size of the gene networks grew. Some recent efforts have taken a less mechanistic, more phenomenological approach: For example, Nielsen *et al.* developed a software package, Cello, that automates the design of biological circuits whose desired behavior is expressed in the digital logic design language Verilog (*87*). Of the 60 circuits that they designed in *E. coli*, 45 performed correctly for every predicted output. Cello builds circuits by using a library of genetic modules with well-characterized input-output relationships, combining modules together by mapping the output

range of one module to the input range of another. Notably, circuit predictions became more accurate after incorporation of constraints to exclude combinations of modules that behaved unpredictably as well as mechanisms to insulate individual modules.

This phenomenological approach parallels recent progress in modeling and predicting the behavior of mammalian gene circuits from Davidsohn et al. (*88*), who adopted a hybrid phenomenological and mechanistic strategy for modeling transcriptional cascades in transiently transfected mammalian cells. Because such experimental systems never reach a steady state, Davidsohn et al. used a set of rate functions to model the production and loss of each transcriptional product. By characterizing several input-output relationships of regulatory parts and then keeping track of their evolution over time, the investigators achieved predictions with a 1.6-fold mean error over a 261-fold range in output. Such a hybrid approach may enable more-advanced and -predictive biodesign and modeling tools for therapeutic gene networks.

Finally, a key hurdle to therapeutic deployment of synthetic gene circuits is safe and efficient delivery into a patient's body. One promising approach for delivery directly into patient cells is to use adeno-associated virus (AAV) because it efficiently delivers genetic material to cells in the human body with minimal immune response (*3*). Although AAV has been used in several recent gene therapy clinical trials (*3*), the nucleic acid packaging capacity of AAV is only approximately 5 kb (*89*), which is too small for many of the synthetic gene circuits described above (although AAV codelivery is being investigated). Other viral vectors, such as HSV-1, have a much larger packaging capacity, well over 100 kb, but their immunogenicity limits their applications (*90*).

A number of alternative nucleic acid delivery methods are also under development. One approach is to introduce purified DNA or mRNA into cells using physical forces, such as electroporation, or synthetic carriers, such as zwitterionic lipids or cationic polymers (*91*). These methods are not subject to the same packaging-capacity limits as viral vectors and, furthermore, can be produced in a completely cell-free manner, simplifying the manufacturing process and reducing the risk of unexpected contaminants in the final product. Unfortunately, nonviral delivery systems have their own challenges: Mechanical methods such as electroporation work well in vitro but are difficult to use in human subjects, and synthetic carrier-based delivery of large nucleic acids often triggers an undesirable immune response (*65*). Chemically modified nucleic acid vectors and biodegradable synthetic carriers that have reduced toxicity owing to their rapid elimination represent a promising step forward in advancing these systems into the clinic (*65*, *92*).

By contrast, engineered-cell therapies deliver genetic material to cells ex vivo and then use the engineered cells as "living therapeutics." In methods based on adoptive cell transfer, a patient's own cells are extracted, engineered, and expanded in a laboratory, then transplanted back into the patient. This approach allows for efficient ex vivo delivery of genetic material and has seen recent successes in clinical trials, including CAR T cell–based cancer therapies (*2*) and engineered hematopoietic stem cells used to treat β-thalassemia (*1*) and adenosine deaminase severe combined immunodeficiency (*93*). Alternately, genetically engineered cells can be microencapsulated in a biocompatible polymer matrix, such as alginate, and transplanted directly into the body (*67*), as described in the diet-induced obesity and psoriasis circuit examples above. Because the encapsulated cells do not provoke an immune response, these prosthetic genetic circuits can be tested and optimized in vitro in the same cell line in which they will operate in vivo, increasing the likelihood that the therapeutic circuit will function as desired.

More broadly, synthetic biology–based therapies carry the risk that either the delivery vector or the proteins expressed by the gene may cause T cells to become reactive or induce so-called antidrug antibodies (ADAs) (*94*). The consequences of an immune response can vary from a reduction in therapeutic efficacy all the way to a life-threatening reaction. For example, a T cell response to nonhuman proteins in ex vivo engineered-cell therapies may cause those cells to be destroyed, blunting the efficacy of the therapeutic. On the other hand, ADAs against human proteins or nonhuman proteins expressed by gene therapies in situ could lead to a severe autoimmune response. To address these issues, strategies have been developed to suppress or mitigate the immune responses against therapies. For example, administration of corticosteroids was used to dampen the T cell response to AAV capsids in a clinical trial in which factor IX was expressed in hemophilia B patients (*95*). Notably, the expression levels of factor IX were sustained for several years in those patients who were promptly treated with steroids after a T cell response (*96*). More recently, Kishimoto et al. coadministered therapeutic biologics and poly(lactic-co-glycolic acid)–encapsulated rapamycin nanoparticles to mice and nonhuman primates to induce immunological tolerance toward the biologics and prevented antidrug immune responses (*97*, *98*). A recent clinical trial used this strategy to induce tolerance against a yeast uricase enzyme for the treatment of gout (*99*). In the future, it may be possible to encode mechanisms to prevent antidrug immune responses in therapeutic gene circuits themselves.

The rapid development of our ability to manipulate biological systems using synthetic genes has direct implications for medicine. More than two decades after the first gene therapy trial was initiated (*100*), we have witnessed several regulatory approvals of gene- and engineered-cell–based therapies (*101*, *102*). The advancement of gene and engineered-cell therapies into the clinic brings with it opportunities for synthetic biologists to create new treatments using synthetic gene circuits. These circuits promise to make gene and engineered-cell therapies both safer and more effective as well as enable treatment options for diseases, genetic and otherwise, that are currently intractable. Taken together, these prospects will continue to propel synthetic biology into the clinic, where it can have a major impact on human health.

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Programming gene and engineered-cell therapies with synthetic biology

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Toward programmed therapeutics

Advances in synthetic biology are enabling the development of new gene and cell therapies. Kitada *et al.* review recent successes in areas such as cancer immunotherapy and stem cell therapy, point out the limitations of current approaches, and describe prospects for using synthetic biology to overcome these challenges. Broader adoption of these therapies requires precise, context-specific control over cellular behavior. Gene circuits can be built to give sophisticated control over cellular behaviors so that therapeutic functions can, for example, be programmed to activate in response to disease biomarkers.

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